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## **Inflammatory chemokines and metastasis – tracing the accessory**

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## Inflammatory chemokines and metastasis – tracing the accessory

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## **Abstract**

The tumor microenvironment consists of stromal cells and leukocytes that contribute to cancer progression. Cross-talk between tumor cells and their microenvironment is facilitated by a variety of soluble factors, including growth factors, cytokines such as chemokines. Due to a wide expression of chemokine receptors on cells in the tumor microenvironment, including tumor cells, chemokines affect various processes such as leukocyte recruitment, angiogenesis, tumor cell survival, tumor cell adhesion, proliferation, vascular permeability, immune suppression, invasion and metastasis. Inflammatory chemokines are instrumental players in cancer-related inflammation and significantly contribute to numerous steps during metastasis. Recruitment of myeloid-derived cells to metastatic sites is mainly mediated by the inflammatory chemokines CCL2 and CCL5. Tumor cell homing and extravasation from the circulation in distant organs are also regulated by inflammatory chemokines. Recent experimental evidence demonstrated that besides leukocyte recruitment, tumor cell-derived CCL2 directly activated endothelial cells and together with monocytes facilitated tumor cell extravasation, in a CCL2- and CCL5-dependent manner. Furthermore, CX3CL1 expression in the bone facilitated metastasis of CX3CR1 expressing tumor cells to this site. Current findings in preclinical models strongly suggest that inflammatory chemokines play an important role during metastasis and targeting of the chemokine axis might have a therapeutic potential.

## **Introduction**

Epidemiological studies have demonstrated that chronic inflammation predisposes individuals to development of various types of cancer, defining inflammation as a hallmark of cancer (1). Enhanced presence of inflammatory cells and soluble inflammatory mediators, such as cytokines and chemokines in the primary tumor are mostly associated with poor prognosis due to metastasis (2-4). However, the immune system has a dual role in cancer progression that depends on the cellular context of infiltrating cells and the tumor type (5). Chemokines and chemokine receptors play a key role in cancer-associated inflammation, where the chemokine-chemokine receptor axis affects both the composition and the function of cells within the tumor microenvironment (4, 6, 7). While the contribution of persistent inflammation to carcinogenesis is already recognized as a hallmark of cancer (1, 8, 9), there is accumulating evidence that chemokines significantly contribute to cancer progression and metastasis through distinct mechanisms (4, 10).

Chemokines are chemotactic cytokines with many functions that are best known for their ability to induce directed migration of cells. Chemokines are produced by a number of different cells in response to cytokines (e.g. TNF superfamily members) and growth factors (e.g. TGF, PDGF). Their functions encompass movement of cells during inflammation, specific recruitment of lymphocytes, dendritic cells, macrophages and stem cells during leukocyte homing, organogenesis as well as cell polarization. Directed migration of cells expressing specific chemokine receptors occurs along a chemokine gradient towards higher local concentrations of ligand. Based on the cellular context and the site of expression, chemokines can be divided into “inflammatory chemokines” (e.g. CCL2, CCL5) which facilitate recruitment of cells during inflammation and “homeostatic chemokines” (e.g. CXCL12, CCL19), which are constitutively expressed in specific tissues (e.g. lymphoid

organs) and predominantly regulate homeostatic trafficking of leukocytes (2, 11). However, some chemokines seem to have dual functions that participate both in immune defenses during inflammation and in trafficking of non-effector leukocytes (12). Development of primary tumors and metastasis is influenced by the complex network of chemokines that regulate leukocyte recruitment and their activation which directly affects tumor growth, invasion and tissue-specific metastasis (2, 13). The role of homeostatic chemokines such as CXCL12, CCL19, and CCL21 in tissue-specific metastasis of different cancer cells expressing the corresponding chemokine receptors CXCR4 or CCR7 is well described and has been reviewed recently (11).

In this review, we focus on the role of inflammatory chemokines and their receptors and summarize the current understanding of their distinct modes of action on stromal and hematopoietic cells during metastasis. We also discuss the potential and the limitations of a possible targeting of chemokines and chemokine receptors in the context of anti-metastatic treatment.

### **Chemokines drive metastasis – initial experimental evidence**

The first report of a chemokine-dependent tissue-specific seeding of cancer cells has been described for breast cancer cells expressing CXCR4 that metastasized to the lung and lymph nodes due to local expression of the homeostatic chemokine CXCL12 (14). Later, the same chemokine axis was identified in melanoma cells (15). CXCL12 is the sole ligand for CXCR4 that is also expressed in the bone marrow (BM) and liver (16, 17). Accordingly, breast cancer cells metastasize to these organs in a CXCR4-dependent manner.

Another chemokine receptor CCR7 has been implicated in melanoma metastasis to lymph nodes (18). Its two ligands, homeostatic chemokines CCL19 and CCL21, are preferentially expressed in secondary lymphoid tissues, and CCL19 is also expressed in the brain (19). CCL19 expression by endothelial cells in the brain is essential for CCR7-expressing T-ALL leukemic cells to specifically seed and to metastasize to this tissue (20). Since the initial reports on homeostatic chemokines as facilitators of tissue-specific metastasis, several other tumor cells from prostate, lung, colorectal, gastric and brain cancer were shown to express corresponding chemokine receptors that facilitate metastasis (11). In contrast, the expression of inflammatory chemokines is induced by an inflammatory trigger that could be derived either from tumor cells or stromal cells (21). Recent clinical evidence together with preclinical data identified several inflammatory chemokines (CCL2, CCL5, CXCL8, CX3CL1) as promoters of cancer progression and metastasis.

### **Inflammatory chemokines and cancer - clinical evidence**

CCL2 and CCL5 are connected with cancer progression and particularly with metastatic disease. High levels of CCL2 and/or CCL5 have been associated with a poor outcome and shorter disease-free survival after surgery due to high incidence of metastasis in patients with breast, colon, prostate, and cervical cancer (22-27). In breast cancer high expression levels of CCL2 were a significant indicator of an early relapse (22). As a consequence, increased infiltration of tumor-associated macrophages resulted in higher vessel density due to angiogenesis (22, 28). In addition, CCL5 was found to be a predictive factor for stage II breast cancer patients and the co-expression of CCL2 and CCL5 in the same tumor was associated with a more advanced stage of the disease (23, 29). Microarray analysis of 2,254 human breast samples identified CCL5-CCR5 signaling to be preferentially active in the basal and

HER-2<sup>+</sup> breast cancer subtypes (24). Importantly, CCR5 expression correlated with enhanced invasiveness and increased metastasis, and consequently, poor patient survival. Similarly, high CCL2 expression levels in colorectal cancer were linked to multiple enhanced liver metastases, and thereby, to poor prognosis for patients (25). In prostate cancer, CCL2 was produced in high amounts when compared to healthy epithelium (26). Interestingly, immunohistochemical staining of CCL2 revealed that not only tumor cells, but also surrounding stromal cells produced high levels of CCL2, suggesting autocrine and paracrine stimulation within the tumor microenvironment. CCL2 expression levels in prostate cancer correlated with the degree of tumor aggressiveness determined by Gleason score (26). In cervical cancer, the absence of CCL2 expression was associated with relapse-free survival (27). In all analyzed cancer types, CCL2 expression was associated with increased infiltration of tumor associated macrophages, which are known to be important for tumor progression, growth and angiogenesis.

Some pancreatic and prostate cancers are spreading by perineural invasion that is defined as the presence of cancer cells in the perineural space (30). Prostate cancer cells express high levels of CX3CR1 compared to normal prostate epithelial cells which specifically binds to the transmembrane chemokine CX3CL1 expressed on neurons, nerve fibers and activated endothelium (31). In prostate cancer, enhanced CX3CR1 expression correlated with bone metastasis while in breast cancer CX3CR1 expression predicted occurrence of brain metastasis (32, 33).

CXCL8 (IL-8) is a pro-inflammatory chemokine that is associated mainly with melanoma (34). CXCL8 expression levels in melanoma tumors and serum CXCL8 levels have been shown to correlate with tumor progression in melanoma patients (34, 35).

Acute Lymphoblastic Leukemia (ALL), a frequent childhood malignancy, is caused by genetic abnormalities. However, the mechanism providing a selective advantage for leukemia cells in the BM remains elusive. Recent findings indicate that bone-marrow plasma levels of CCL2 and CXCL8 are significantly elevated in patients at diagnosis compared to controls (36). Increased levels of CCL2 and CXCL8 in the bone marrow microenvironment enhanced adhesion of leukemia cells to the BM mesenchymal stem cells, and promoted their survival. CCL2 levels were also significantly increased in ALL patients with involvement in the CNS, and were further elevated during chemotherapy (37). However, further clinical evaluations are needed to assess the role of inflammatory chemokines during cancer progression.

### **The chemokine-chemokine receptor axis forms the tumor microenvironment**

The tumor microenvironment at primary or metastatic sites can be actively shaped by tumor - derived chemokines, resulting in the recruitment of leukocytes and activation of pro-inflammatory mediators (1, 23, 38). It is currently accepted that inflammatory chemokines contribute to cancer progression by several mechanisms including: recruitment of inflammatory cells, immune suppression, angiogenesis and metastasis (Figure 1). While activation of chemokine receptors on stromal cells including leukocytes indirectly support cancer progression, engagement of chemokine receptors on tumor cells can directly influence their behavior and invasive capabilities.

### **Chemokine receptors on tumor cells – influence the metastatic behavior**

Tumor-derived chemokines have been shown to directly affect tumor cells in an autocrine manner. Murine breast cancer cells expressing high levels of CCL5 spontaneously



metastasized to lungs and liver (39). Knock-down of CCL5 in 4T1 cells resulted in a strong reduction of metastasis and correlated with a reduced expression of MMP-2 and MMP-10. These findings suggested that CCL5 directly affected the metastatic capacity of 4T1 cells through modulation of MMP production. High-grade bladder cancer cells expressing high levels of CCL2 mediated their own increased migration and invasive capacity, when compared to low-grade bladder cancer cells expressing low CCL2 levels (40). Inhibition of CCR2 resulted in reduced invasiveness of bladder cancer cells and attenuated metastasis. Recent studies have shown that CCR2 overexpression in prostate cancer correlated with poor prognosis and that CCL2 signaling promoted tumor cell survival and metastasis to the bone (7, 41). This finding was further supported by the observation that CCL2 treatment of PC3 prostate cancer cells protected them from nutrient-induced autophagic death through activation of the PI3K/Akt/survivin pathway (42). In breast cancer cells, up-regulated CCR2 expression corresponded with increased CCL2 expression and resulted in improved survival (43). CCR2 knockdown in breast cancer cells significantly attenuated CCL2-driven cell migration and survival. Human malignant melanomas express and secrete high amounts of CXCL8 which correlated with enhanced metastasis (44). The majority of melanomas express CXCR2, the receptor for CXCL8; this has been shown to directly stimulate metastatic outgrowth (45). As expected, neutralization of CXCL8 attenuates experimental metastasis (46).

### **Chemokines and angiogenesis**

Chemokines are critical mediators of neovascularization in many physiological and pathophysiological situations including cancer progression (47). Chemokines of the CXCL family can be divided into proangiogenic chemokines or angiostatic chemokines based on a

unique three amino acid sequence ELR. ELR-positive chemokines (e.g. CXCL1, CXCL2, CXCL8) promote angiogenesis by engaging the CXCR2 on endothelial cells (48). CXCR2 has a critical role in neovascularization, since a CXCR2-neutralizing antibody blocked the proangiogenic activity of the CXCL8. In contrast, ELR-negative chemokines (e.g. CXCL4, CXCL9, CXCL10, CXCL11) have an angiostatic activity that is mediated through the CXCR3 on mononuclear cells (49).

In addition to CXCL chemokines, CCL2 was reported to stimulate angiogenesis and chemotaxis of human microvascular endothelial cells *in vitro*, providing evidence for CCR2 expression on endothelial cells (50, 51). Whether or not CCL2 directly stimulates angiogenesis remains controversial. It is believed to be dependent on the tissue origin of endothelial cells (50, 52). Direct stimulation of prostate cancer cells with CCL2 resulted in induced expression of pro-angiogenic factors like VEGF, suggesting an additional indirect effect on angiogenesis (53). Interestingly, the transcription factor Twist1 that induces epithelial to mesenchymal transition also promoted angiogenesis in mammary epithelial cells through CCL2-mediated recruitment of macrophages (54). Similarly, direct induction of VEGF expression and thereby angiogenesis has been proposed to be dependent on CCL2 production and macrophage recruitment (22). Increased recruitment of F4/80<sup>+</sup> macrophages to FGFR1-dependent breast cancer promoted angiogenesis in the mammary gland, which was dependent on CX3CL1 (55). These results support the notion that chemokine-recruited macrophages produce a number of cytokines that affect cancer progression including angiogenesis.

## **Immune suppression is modulated by CCL2**

Different tumors require various mechanisms to sustain their growth and to establish a suitable microenvironment for their persistence. Along this line, a mechanism of immune escape in melanoma has been documented to be CCL2-dependent (56, 57). Recruitment of monocytic CCR2<sup>+</sup> myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment was shown to promote tumor growth through immune suppression (56). Ablation of CCR2<sup>+</sup> MDSCs resulted in increased infiltration of activated cytotoxic CD8<sup>+</sup> T-cells and reduced tumor growth. In non-small-cell lung cancer, the use of anti-CCL2 antibody significantly slowed down tumor growth and also inhibited spontaneous lung metastasis (57). Although no major difference in numbers of tumor-associated macrophages was detected, changes in their polarization status resulted in activation of CD8<sup>+</sup> T-cells (57).

## **CCL2-CCR2 anti-metastatic activity**

In contrast to the well-documented function of the CCL2-CCR2 axis in promoting metastasis, there are reports describing the opposite effect resulting in inhibition of tumor progression and metastasis (58, 59). Host-derived CCL2 and CCL3 recruited CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and NK cells that controlled tumor progression and metastasis (60). Lung metastasis was enhanced by the deficiency of CCL2 and CCL3. Overexpression of CCL2 in 4T1 breast cancer cells resulted in reduced metastasis. However, the mechanism remained to be described (58). In an orthotopic breast cancer model using 4T1 cells, CCL2 had a pro-tumorigenic activity at primary sites, while it inhibited metastasis to the lungs (59). Since down-regulation of CCL2 in tumor cells resulted in reduced primary tumor growth but accelerated metastasis, CCL2 was suggested to be essential for the entrainment of tumor-associated neutrophils. Indeed, neutrophil depletion had no effect on the growth of primary

tumors, but resulted in enhanced lung metastasis indicating that neutrophils have a protective anti-metastatic trait. The switch between these anti- and pro-tumorigenic phenotypes was regulated by tumor cell-derived TGF $\beta$  (59, 61). However, there is conflicting data on the role of granulocytes/neutrophils during metastatic initiation. Kowanetz and colleagues showed that Ly6G<sup>+</sup>Ly6C<sup>+</sup> cells (granulocytes) are recruited to the pre-metastatic lung by tumor-secreted G-CSF in 66c14 (a 4T1 sibling cell line) tumor-bearing mice (62). Depletion of granulocytes with anti-Gr1 or anti-Ly6G antibody reduced the number of lung metastases indicating that Ly6G<sup>+</sup>Ly6C<sup>+</sup> cells contribute to the generation of lung metastases. Taken together, the role of CCL2 and respective CCR2<sup>+</sup> cells during tumorigenesis and metastasis appears to be strongly dependent on the cellular composition, the tumor type and the stage of disease. In addition, the use of different mouse models and tumor cells are reflected in seemingly opposite observations. Being aware of these limitations, further experiments are required for dissection of the potentially ambivalent role of CCL2 during metastasis.

### **Chemokines regulate leukocyte recruitment to metastatic sites**

Formation of a chemokine gradient on proteoglycans presented either in the extracellular matrix or on the endothelium is a prerequisite for leukocyte recruitment. Suppression of expression of the proteoglycan versican, led to reduced CCL2 binding and attenuation of metastasis (63). Versican promoted metastasis through enhancement of tumor-promoting inflammatory responses in the lungs and revealed CCL2 as a key factor in this process.

Studies in various animal models confirmed that high expression levels of CCL2 and CCL5 correlated with metastasis (64-68). Accordingly, the recruitment of CCR1-, CCR2-, and CCR5-positive monocytes and myeloid cells was associated with cancer progression. CCL5 expression in breast cancer cells was linked to infiltration of CCR1- and CCR5-positive

leukocytes and promoted tumor growth (65, 69). The use of the CCR1 and CCR5 antagonist Met-CCL5 significantly reduced tumor growth of already established tumors (65). Further studies indicated concomitant expression of CCL2 and CCL5 in breast cancer cells that was associated with cancer progression and metastasis (23).

In another model, overexpression of CCL2 in MDA-MB-231 human breast cancer cells promoted experimental metastasis to the lungs and the bone, while the use of neutralizing anti-CCL2 antibodies significantly reduced metastasis to these organs (64). Moreover, monocytic stromal cells expressing CCR2 contributed to breast cancer metastasis that was dependent on CCL2 produced from both tumors and stroma. The recruitment of circulating monocytic cells to metastatic sites has been identified as a critical factor for breast, colon, and lung cancer dissemination (66, 67). CCR2<sup>+</sup> inflammatory monocytes were shown to promote tumor extravasation (66). Recently, we confirmed the critical role of CCR2-expressing inflammatory monocytes (CD11b<sup>+</sup>, Ly6C<sup>hi</sup>) that are recruited to metastasizing tumor cells (MC-38GFP colon or Lewis lung carcinoma 3LL) and facilitate efficient tumor cell extravasation (67). Intravenous injection of these cells in mice with specific depletion of CCR2 in the myeloid cell population (LysMCre*Ccr2*<sup>loxP/loxP</sup>) resulted in reduced metastasis, directly confirming the essential role of CCR2<sup>+</sup> monocytes during metastasis. In another study, a distinct population of CCR2<sup>+</sup>/CD11b/Gr1<sup>mid</sup> cells that promote experimental liver metastasis were identified, based on a significant reduction of metastasis after depletion of CD11b/Gr1<sup>mid</sup> cells (68). Interestingly, experimental metastasis of MC-38GFP and 3LL was found to be dependent on CCL2 expression by tumor cells (67, 68). Specific down-regulation of CCL2 in both MC-38 and 3LL cells resulted in a significantly reduced metastatic capacity, suggesting that direct interaction of the tumor cell-derived chemokine with monocytes is necessary for efficient tumor cell extravasation (67). In contrast, metastasis of B16-BL6

melanoma cells was independent of CCR2 expression, suggesting that some tumor cells use other means for tumor cell extravasation which remain to be determined (67, 68).

These observations compellingly showed that CCL2 and CCL5-dependent recruitment of myeloid cells contribute to tumor cell colonization of distant organs including lungs, bones, and liver. However, the exact function of these cells during metastasis requires further analysis in the context of the metastatic microenvironment; the formation of which may already begin during the hematogenous phase.

### **Tumor cell extravasation is dependent on endothelial CCR2 expression**

Recently, a novel role for the chemokine receptor CCR2 in facilitating tumor cell extravasation has been described (67). Significantly less lung metastasis developed in *Ccr2*<sup>-/-</sup> mice compared to wild-type mice using an experimental metastasis model. The lack of stromal CCR2 expression led to a reduced lung tumor burden compared to control mice. This suggested an important role for endothelial CCR2 expression in metastasis (Figure 2). Mice expressing CCR2 exclusively on endothelial cells under the Tie2 promoter consequently showed only a mild attenuation of metastasis compared to wild-type controls but had significantly more metastatic foci compared to *Ccr2*<sup>-/-</sup> mice. Furthermore, increase of vascular permeability and subsequent tumor cell extravasation was shown to be dependent on endothelial CCR2 expression. These observations were further confirmed in assays with primary lung endothelial cells isolated from wild-type and *Ccr2*<sup>-/-</sup> mice *in vitro*. Tumor cell transmigration through CCR2-deficient endothelial cells was significantly reduced, indicating the indispensable role for endothelial CCR2 in facilitating tumor cell transmigration and metastasis.

Inhibition of JAK2, the direct downstream mediator of CCR2, abolished the induction of lung permeability, inhibited tumor cell transmigration in the *in vitro* setting and attenuated metastasis (67). Downstream signaling of JAK2 was mediated through Stat5 and p38MAPK, the inhibition of which reduced lung vascular permeability and tumor cell extravasation. This was in agreement with previous findings that p38MAPK pathway is activated in endothelial cells by transmigrating tumor cells (70). However, the activation of the p38MAPK pathway as a consequence of a chemokine-chemokine receptor interaction has not been previously reported. Several studies are pointing towards an increased aggressiveness of various tumor types, including colon carcinoma and prostate cancer, when Stat5 signaling is constitutively activated in the tumor (71, 72). However, the role of Stat5 activation in promoting metastasis has not been previously assessed.

In conclusion, the finding that endothelial CCR2 determines the efficacy of tumor cell extravasation indicates that CCR2-dependent recruitment of monocytic cells follows the initial increase in vascular permeability and enables efficient metastasis. It is likely that expression of CCR2 on endothelial cells during metastasis makes the inhibition of CCR2 a better target than inhibition of CCL2, which is present not only within the tumor but also in the circulation in significantly larger amounts. However, the dynamics of endothelial CCR2 expression not only in the lungs but also other tissues remains to be determined.

### **Stromal-derived chemokines regulate metastasis**

The cross-talk between tumor cells and the surrounding stroma is at least in part, mediated by chemokines that directly affect tumor progression. CCL2 produced by cancer-associated fibroblasts has been shown to regulate stromal-epithelial interactions in a breast cancer model (73). Targeted ablation of TGF $\beta$  signaling in fibroblasts resulted in enhanced CCL2 secretion

and increased recruitment of tumor-associated macrophages. The resulting increase in tumorigenesis and metastasis was abrogated with CCL2-neutralizing antibodies (73). Similarly, fibroblast-specific knock-down of CCL2 led to attenuated outgrowth of human breast cancer cells (74).

During the hematogenous phase of metastasis, tumor cells also induce expression of chemokines by locally activated endothelium (75). Tumor cell-induced platelet-aggregation in the circulation promotes the arrest of metastasizing tumor cells in the vasculature and results in local endothelial activation and induced CCL5 expression *in vivo*. This process is facilitated by selectin-mediated interactions of tumor cells with platelets and leukocytes and further led to CCL5-dependent recruitment of circulating monocytes. Temporal inhibition of the CCL5 axis attenuated metastasis of both mouse and human colon carcinoma cells in an experimental metastasis mouse model (75).

Another cell population contributing to breast cancer metastasis is mesenchymal stem cells (MSCs) (76). MSC-derived CCL5 production directly affected tumor cells which led to a significant increase in the metastatic behavior of otherwise poorly metastatic cells. Further analysis identified CCL5 as a factor enabling efficient extravasation of tumor cells.

### **Chemokines and the metastatic niche**

There is accumulating evidence that tumor-derived factors enable mobilization of BM-derived cells, which in the context of certain tissues contribute to the formation of a pre-metastatic niche (77). BM-derived hematopoietic progenitor cells were shown to be recruited to the pre-metastatic niche and to form cellular clusters before the arrival of tumor cells (78). The analysis of factors responsible for this process revealed inflammatory chemoattractants of the



S100 family as the major driver for the generation of the pre-metastatic niche (reviewed in 79). The local production of S100A8 and S100A9 at pre-metastatic sites (e.g. endothelial and myeloid cells) is induced by factors released from primary tumors (80). One of the factors derived from the primary tumors is CCL5 which induced the release of S100A4 by stromal and tumor cells resulting in up-regulation of cytokines and chemokines (including G-CSF, CXCL5, CXCL3, CCL20, CCL5, CCL9) in tumor cells (81). CCL5-expressing tumor cells showed a significant reduction in lung metastasis in S100A4<sup>-/-</sup> mice, while liver metastasis was not affected, indicating that at least metastases to the lungs are highly dependent on the interplay between CCL5 and S100A4.

Findings of tumor-derived factors responsible for the formation of a pre-metastatic niche raised the question of how specific tissues are targeted. Tumor-derived microvesicles, so-called exosomes, were identified as transporters of stimulating factors to future metastatic sites (82). Melanoma-derived exosomes have been shown to induce lung vascular permeability and inflammation, thus generating a pre-metastatic niche in the tumor-targeted organ (83). Tumor-derived exosomes recruited MDSCs to the lungs of tumor bearing mice in a MyD88-dependent manner and thereby increased the metastatic burden (84). In addition, platelet-derived microvesicles have been shown to increase the metastatic potential of tumor cells (85). These data further support the notion that alteration in the tissue microenvironment may lead to pre-metastatic niche formation and promote metastasis to activated tissues. Whether and how inflammatory chemokines contribute to the formation of the pre-metastatic niche remains to be defined.

The role of CCL2 in the development of bone metastasis involved CCR2<sup>+</sup> stromal cells which promote the development and maintenance of bone metastases of prostate and breast cancer origin. Chemotaxis of prostate cancer cells is driven by CCL2, suggesting the possible

involvement of CCL2 in tissue specific migration to the bone (26, 86). In addition, an involvement of receptor activator of NF- $\kappa$ B ligand (RANKL) and CCL2 was reported in osteoclastogenesis and subsequent bone resorption that enabled metastasis of prostate cancer to the bone (41, 87). Similarly, CCL2 enhanced migration of breast cancer cells and has been suggested to mediate homing of breast cancer cells to the bone (88). However, these studies rely on the examination of prostate cancer cell lines and conditioned medium *in vitro*. Furthermore, prostate cancer-stimulated osteoblastic CCL2 production in the bone microenvironment was shown to be required for metastatic outgrowth (53). Mice carrying subcutaneous tumors of different prostate cancer cells induced CCL2 expression in the BM microenvironment which was mediated by tumor-derived parathyroid hormone-related protein secretion (53). The role of CCL2 on osteoclastogenesis was demonstrated in mice which were injected with prostate cancer cells in the tibia (53). Tumor growth in the bone was significantly attenuated by anti-CCL2 antibody treatment. Cyclophosphamide treatment of prostate cancer-bearing mice induced specific upregulation of cytokines and chemokines, including CCL2, in the BM that resulted in preferential recruitment of myeloid cells to these sites (89). The subsequent increase in bone metastasis could be reduced by anti-CCL2 neutralizing antibody treatment. This study provided evidence that BM perturbation due to cytotoxic therapy may contribute to bone metastasis through a transient disruption of the BM vascular integrity and expansion of monocytes and neutrophils in the circulation.

CX3CR1 expression on pancreatic and prostate cancer increased their invasiveness and specific metastasis to the neuronal tissues (30). The unique transmembrane expression of CX3CL1 on the cell surface of neurons and activated endothelium enabled also tumor cell adhesion (31). Overexpression of CX3CR1 in human pancreatic cells correlated with enhanced neuronal invasiveness in mice. The correlation between enhanced CX3CR1 expression and bone metastases was shown also in breast cancer (90). The bone metastatic

cell line MDA-MB-231 expressed significant levels of CX3CR1 while MDA-MB-436, which does not metastasize to the bone, had undetectable levels. Experimental bone metastases of MDA-MB-231 cells were significantly reduced in CX3CL1-deficient mice (90). Functional CX3CR1 expression mediated adhesion of tumor cells to the endothelium since adhesion-deficient mutant of CX3CR1 abrogate tumor cell recruitment to the bone.

In summary, the contribution of chemokines to metastasis largely depends on the tissue at metastatic sites and cross-talk within the local microenvironment. Inflammatory chemokines promote tissue-specific metastasis and the current experimental evidence clearly links inflammatory chemokines to the formation of the metastatic niche which promotes tumor outgrowth in the lungs, liver and bone.

### **Current concepts and future directions**

Efficient prevention of metastatic tumor spread or eradication of already existing metastasis has developed into one of the most important scientific, clinical and medical assignments in the last decade of cancer research (1). Efficient inhibition of metastasis by the targeting of tumor cells or specific host cells remains to be achieved. This is primarily due to the complex interplay between various host cell types and tumor cells. The concept of pharmacological inactivation of chemokines or chemokine receptors in order to reduce chemokine-dependent metastasis is under investigation and is not entirely new (7, 91). However, there are various obstacles that make inactivation of chemokines and their receptors *in vivo* a challenge:

- (1) The interactome of chemokines and chemokine receptors is highly complex, with various chemokines binding to one and the same receptor but also various chemokine receptors recognizing the same ligand.

- (2) Inhibition of chemokine signaling may lead to compensatory effects resulting in changes of chemokine profiles (68).
- (3) Inactivation or inhibition of chemokine receptor-expressing cells may have severe consequences, due to the wide range of cells expressing chemokine receptors, although a time-defined treatment may be tolerated.
- (4) Since metastasis is a cascade of sequential and interlinked events, inactivation of specific chemokine-chemokine receptor functions may only be successful within a restricted time window.

Thus, there is urgent need for novel tools (e.g. small molecules, function-blocking antibodies) and additional knowledge to successfully achieve a targeted and efficient inactivation of either chemokines or their receptors. Depending on the cancer stage, the number of circulating tumor cells and the degree of metastasis, a therapy aiming at preventing tumor cell extravasation may strongly reduce the risk of further cancer dissemination, but may not affect already established deposits. Thus, therapeutic approaches to inhibit chemokine receptor signaling for prevention of metastasis and to limit outgrowth of already established ones should be considered in future. Therefore, the goal will most likely be a combination therapy with targeting also tumor growth.

Despite these open questions, inhibition of CCL2 or CCR2 was reported to be beneficial in inhibiting metastasis of various cancer types such as breast, bladder, colorectal or prostate cancer in experimental models (66-68). Based on this preclinical evidence, two clinical trials have been started, evaluating safety and efficacy of blocking both CCL2 and CCR2 in metastatic patients (<http://www.clinicaltrials.gov>: NCT00992186, NCT01015560). The initial trial for the treatment of patients with metastatic castration-resistant prostate cancer used the antibody “CNTO 888” which blocks CCL2. However, blocking CCL2 proved to be

less effective possibly due to induced compensatory mechanisms that led to an increase in CCL2 expression (68). In a second ongoing phase II trial, the anti-CCR2 antibody “MLN1202” is being used for treatment of patients with bone metastasis. Importantly, currently there is no anti-metastatic therapy clinically available, therefore additional criteria assessing efficacy will be required. Moreover, future studies will show whether additional chemokine-chemokine receptor pairs can be targeted for clinical evaluation.

### **Conflict of interest**

Authors declare no conflict of interest

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## Figure legends

### **Figure 1: Direct effects of chemokines on the tumor microenvironment during cancer progression**

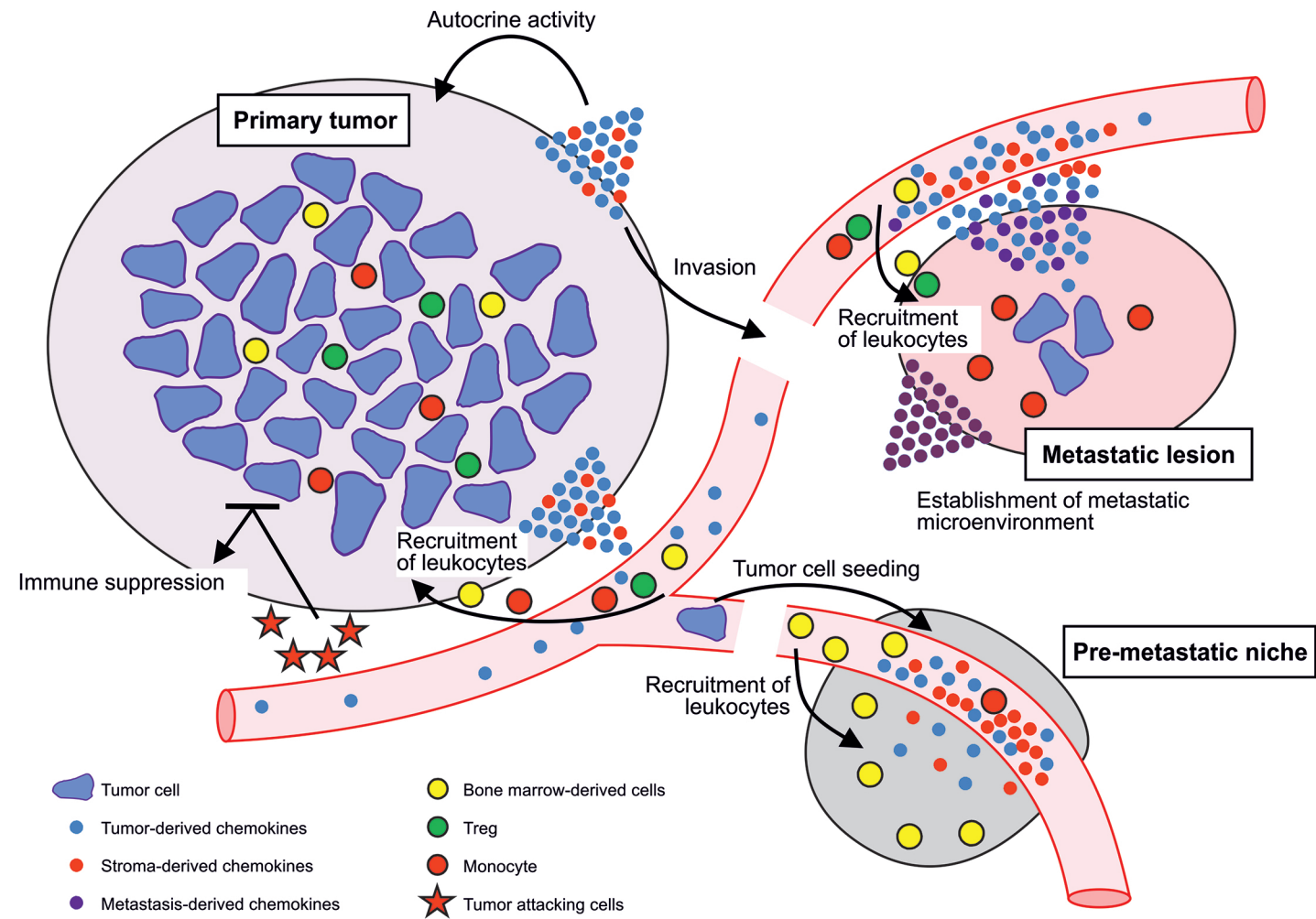
Chemokines secreted by tumor and stromal cells at primary tumor sites generate a chemokine gradient, which is required for the recruitment of leukocytes. Leukocyte recruitment, predominantly of myeloid-derived cells, supports tumor survival and helps to establish an immune-suppressive microenvironment. This tumor-protective environment hinders recruitment of tumor-attacking cells and causes their functional non-responsiveness. In addition, chemokines also have autocrine functions and may lead to acquisition of an invasive phenotype of tumor cells, a pre-requisite for the spread of tumors to distant organs. Prior to tumor cell arrest, formation of a pre-metastatic niche by BM-derived progenitor cells promotes metastatic seeding to certain organs. As tumor cells reach the targeted organ via the vascular system, they arrest on endothelial cells and trigger an inflammatory reaction. Induction of tumor cell extravasation is dependent on CCR2-mediated endothelial activation. This is followed by production of chemokines by tumor, endothelial and stromal cells and a recruitment of monocytic CCR2<sup>+</sup> cells, enabling efficient tumor cell extravasation. At new sites, the cycle of chemokine gradient generation and recruitment of leukocytes leads to the generation of a metastatic microenvironment. This further promotes tumor cell proliferation and leads to the generation of metastatic lesions.

### **Figure 2: CCL2-CCR2 mediated mechanisms in tumor cell extravasation**

**A)** Tumor cell-derived CCL2 leads to the recruitment of CCR2<sup>+</sup> monocytes. Upon binding to endothelial CCR2, endothelial cells become activated and various signaling pathways are triggered. **B)** Triggering of endothelial CCR2 by CCL2 leads to phosphorylation of JAK2 which subsequently can activate various downstream signaling pathways. Signaling through

Stat5 and p38MAPK pathways is important for tumor cell extravasation whereas Stat3, PI3K and Rac1 activation seems not to be involved. C) Activation of endothelial cells via the CCL2-CCR2-axis leads to cytoskeletal retraction within endothelial cells resulting in induction of vascular permeability. Disruption of the endothelial layer and gap formation between endothelial cells then allows transmigration of tumor cells together with monocytes. However, the exact dynamics and kinetics of cell-cell interactions promoting transmigration remain to be determined.

**Figure 1**



**Figure 2**

